*Medical Journal of Babylon*
*Vol. 13- No. 2: 337 - 346 , 2016*
*http://www.medicaljb.com*
*ISSN 2312-6760©2016 University of Babylon*

*Original Research Article*

# Relationship between Thyroid Function, Cystatin C and Different Oxidative Stress in Iraqi Patients with Chronic Kidney Disease

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Accepted 27 March, 2016

## Abstract

Chronic Kidney Disease (CKD) is associated with slightly higher frequency prevalence of primary hypothyroidism , but at the same studies on thyroid hormone status in uremic patients has reported conflicting results. This study was undertaken during the period from February 2014 to January 2015to quantify thyroid hormones (T3,T4 and TSH) ,cystatin C, different oxidative stress parameters like serum Ceruloplasmin (CP), Carbonyl, Thiol and total protein (TP) and four trace elements (Molybdenum (Mo), Cadmium (Cd), Manganese (Mn) and Magnesium (Mg) and explore correlation between these parameters in (75) non- dialyzed CKD patients' verses (52) healthy controls. Results indicated that the levels of (T3, T4,CP, TP,thiol,Mo ,Cd and Mg were significantly reduced ($p \leq 0.05$)while the levels of TSH ,cystatin C ,carbonyl and Mnweremildly significantly increase($p \leq 0.05$) in patients group compared to healthy controls.The correlation coefficient ( r ) test is used to describe the association between these parameters,T3 and T4 were negatively correlated with cystatin C ,carbonyl, Mn and Mg, positively correlated with(thiol and protein) .T4 positively correlated with CP while T3 not correlated with CP.TSH positively correlated with (CP, carbonyl,thiol,protein), finally, CP negatively correlated with cystatin C and positively correlated with Mn and Mg.

**Key words:** CKD, antioxidants,cystatin C,ceruloplasmin,Mo,Cd,Mg,thyroid hormones.

## الخلاصة

لوحظ وجود انخفاض في وظائف الغدة الدرقية الابتدائي لدى مرضى الكلى المزمن لذا اجريت هذه الدراسة خلال الفترة من فبراير 2014 وحتى يناير 2015 وتضمنت الدراسة 75 مريض مصابين بامراض الكلى المزمن و52 شخص اصحاء كمجموعة سيطرة وتم فيها قياس مستويات كل من هرمونات الغدة الدرقية ,بعض من مضادات الاكسدة مثل ( انزيم السيريلوبلازمين , الكربونيل , C السيستاتين(T3) ، T4و (TSH) ,الثايول و البروتين الكلي) , اضافة الى تعيين مستوى اربعة من العناصر النزرة ( المولبينوم, الكادميوم, المنغنيز و المغنيسيوم )واخيرا تم تحديد معامل الارتباط المعنوي فيما بين هذه المتغيرات الكيموحيوية .

اظهرت النتائج ان مستويات كل من (T3,T4,CP, thiol,Mo,Cd&Mg) قد انخفضت معنويا بينما لوحظ وجود زيادة معنوية في مستويات كل من TSH, cystatin C ,carbonyl &Mn في مجموعة المرضى مقارنة بالاصحاء. اما نتائج معامل الارتباط المعنوي فأظهرت وجود ارتباط معنوي سالب بين (T4,T3) وكل من cystatin C ,carbonyl, Mn, Mg وارتباط معنوي موجب مع الثايول والبروتين.T4 يرتبط ارتباط معنوي موجب مع السيرلوبلازمين و T3 لا يرتبط ارتباط معنوي مع السيريلوبلازمين .

TSH اظهر ارتباط معنوي موجب مع السيلوبلازمين, الكاربونيل والبروتين واخيرا وجد ان السيرلوبلازمين يرتبط ارتباط معنوي سالب مع السيستاتينC وموجب مع المنغنيز والمغنيسيوم.

**الكلمات المفتاحية :** امراض الكلى المزمنة, مضادات الاكسدة, سيستاتينC ,السيرلوبلازمين, المولبينوم, الكادميوم,المغنيسيوم, هورمونات الثايرويد.

## Introduction

Chronic kidney disease characterizes abnormal kidney function and/or structure. [1].Chronic kidney disease diagnosis based on the presence of kidney damage (i.e. albuminuria) or decreased kidney function(i.e. Glomerular filtration rate (GFR) <60 ml/minute per1.73 m²) for three months or more, irrespective of clinical diagnosis [2].Patients with CKD often have signs and symptoms suggestive of thyroid dysfunction. Several mechanisms are links between kidney and thyroid function.Thyroid hormones are important for the preserve of electrolyte and water homeostasis (directly by affecting the glomerular /tubular kidney function and the structure of the kidney itself and indirectly by affecting the cardiovascular system and the renal blood flow). Meanwhile, thyroid function abnormalities could represent a risk factor for kidney disease progression[3,4].Serum cystatin C is a non-glycosylated, 13.3-kDa protein belonging to cystatin protease inhibitors. It has shown promise as a replacement for serum creatinine in estimation of GFR[5,6]. Serum cystatinC is released into bloodstream by all nucleated cells,it is freely filtered by kidney glomeruli, metabolized by the proximal tubule and identified as a marker of renal failure[7,8,9].Chronic kidney disease the associated oxidative stress contributes to the progression of renal injury[10].Several antioxidant aimed at modifying the oxidative status in CKDpatients such as Cp (EC:1.16.3.1)serum oxidase activity,a 132kDa copper binding glycoprotein, which is an important extracellular antioxidant, being an acute phase reactant protein [11,12].Carbonyl (CO) groups are produced on protein side chains (especially of Pro, Arg, Lys, and Thr) when they are oxidized[13] and their derivatives can also be generated through oxidative cleavage of proteins by either the amidation pathway or by oxidation of glutamyl side chains, leading to formation of a peptide in which the N-terminal amino acid is blocked by an a-ketoacyl derivative [14].Among all the antioxidants that are available in the body, thiols constitute the major portion of the total body antioxidants and they play a significant role in defense against reactive oxygen species. Thiols are the organic compounds that contain a sulfhydryl group[15]. Total thiols composed of both intracellular and extracellular thiols either in the free form as oxidized or reduced glutathione, or thiols bound to proteins. Among the thiols that are bound to proteins, albumin makes the major portion of the protein bound thiols, which binds to sulfhydryl group at its cysteine-34 portion. Apart from their role in defense against free radicals, thiols share significant role in detoxification, signal transduction, apoptosis and various other functions at molecular level. The thiol status in the body can be assessed easily by determining the serum levels of thiols[16]. Heavy metals include both non-toxic and toxic elements iron (Fe), cobalt (Co), copper (Cu), manganese (Mn), molybdenum (Mo), and zinc (Zn) are the trace elements and they are required in a very minute amount, whereas other metals are non–essential, toxic to animals and fatal when accumulated. For example, mercury (Hg), arsenic (As)/ lead (Pb), plutonium (Pu), vanadium (V), tungsten (W) , magnesium (Mg)and cadmium (Cd)[17].Effect of Motoxicitywere observed in animals include renal failure, reproductive effects, growth depression, and decreased hemoglobin and hematocrit[18].When the renal system is not functioning properly, the clearance of many trace elements is also affected such as Cd and Mg which have been implicate in the decline of the renal functions[19,20].The aim of this study was to estimate the levels of some biochemical parameters includes thyroid hormones, cystatin C, different antioxidantsand four trace elements and made an attempt to investigate the relationship between them in CKD subjects compared to healthy controls.

## Materials and Methods

### Reagents

All laboratory chemicals and reagents were of annular grade.

### Subjects

This study was undertaken during the period from February 2014 to June 2014,the study groups consisted of52 serum samples collected from healthy individuals (20) men and (32) women without any detectable diseases, age range between (20-70) years, and (75)serum samples from patients with chronic kidney disease (30) men and (45) women, age range between (18-75) years. The disease were diagnosed by specialist doctors in AL-Emamain hospital, Baghdad, Iraq.

About 10 ml of vinous blood was collected in plane tube using plastic disposable syringes and left for 20 minutes at room temperature (25ºC). After coagulation, sera were separated by centrifugation at 1500 xg for 15 minutes. Sera were frozen in -20ºC until analysis.

### Determination of T3, T4 and TSH

Triiodothyronine (T3), thyroxin (T4) and thyroid stimulating hormone (TSH) were estimated using VIDAS from Biomerix (France) respectively. The principle of the quantitative determination of T3, T4 and TSH is ELFA technique.

### Ceruloplasmin Activity Assay

The enzymatic assay of ceruloplasmin oxidase activity was carried out using the modified Rice method [21] by using p-phenylene diamine-2HCl as a substrate. The CP activity was expressed in term of (U/L).

### Total Serum Protein

Serum protein concentration was determined by Lowry et. al. method [22], by using bovine serum albumin (BSA) as a standard protein.

### Cystatin-C concentration

Relative concentration of cystatin-C was measured at wavelength of 450 nm using quantitative a sandwich immunoassay (Immunospec-USA ELISA kit). The micro-titer plats contain horseradish peroxidase (HRP)-conjugated polyclonal antibodies specific for cystatin-C.

### Determination of carbonyl levels

Protein carbonyls were estimated using the method of Levine *et al* [23]with slight modifications. Briefly, 0.5 mlserum (1 mg/ml) were treated with 0.5 mltrichloro acetic acid (TCA, 20 % v/v) at room temperature for 10 min, and centrifuged at 4,000 x g. The pellet was treated with 0.5 ml of 10 mM DNPH in 2 M HCl, or with 0.5 ml of 2 M HCl alone for the blank. Samples were incubated for 30 min at room temperature in the dark, and then treated with 20% TCA, and centrifuged at 4,000 x g. The pellet was washed three times in ethanol/ethyl acetate (v/v); and 1.5 mL of 1 M NaOH was added to pellet followed by incubation at 37oC for 15 min. Carbonyl concentrations were determined utilizing molar absorption coefficient of ε370=22,000 M-1cm-1 using a Schimadzu UV-spectrophotometer and expressed as nanomoles of carbonyls per milligram protein.

### Determination of thiol

Serum thiol levels were measured by Ellman reagent (5,5'-ditiobis 2-nitro-benzoic acid- DNTB) as described [24]. Samples were centrifuged at 3,000 x g for 5 min at room temperature. Top phases were decanted and thiol level of all samples were determined by utilizing molar absorption coefficient (14.100 M-1cm-1) using a Schimadzu UV-spectrophotometer and expressed as micromol.

### Determination of serum trace elements

Serum levels of four trace elements(Mo,Cd,Mn and Mg) were determined with flame Atomic Absorption Spectrophotometer (AAS) using a direct method as described by Kaneko [25].

### Statistical Analysis

Statistical analyses were done using Microsoft office(SPSS version 11.4)which includes the following : Mean ± standard deviation , Student t –test , Correlation regression, P value of less than 0.05 was considered significant .

**Results**

The results obtained in this study were from a total number of 127 subjects which Cases(n=75), The mean age of the CKDandcontrol groups were 49±9.1, 52± 5.6, respectively. Demographic characteristics of CKD and control groups are shown in(Table 1).

have been divided into,(Group-1) contain controls (n=52),(Group-2) contain CKD

**Table 1:**Age and sex of CKD and control subjects

| Parameters | Patients (n=75) | Control(n=52) |
|---|---|---|
| **Age(years)** | 49(9.1) | 52(5.6) |
| **Sex(male/female)** | 30/45 | 20/32 |

The results are shown in Table 2, Table 3and Table 4.Statistical analysis was done by unpaired student' s t test. Serumthyroid hormones results showed significantly decrease in the levels of T3 &T4 and significantly increase in the level of TSH in patients group when compared to controls ($p \leq 0.05$),table 2.

**Table 2:**Comparison of serum T3,T4 and TSH between CKD cases and controls

| Groups | T3 ng/ml | T4 µg/ml | TSH mIU/L |
|---|---|---|---|
| **Controls (mean±SD)** | 1.3±0.5 | 0. 86±0.01 | 2.9±0.88 |
| **Cases (mean±SD)** | 0.2±0.098 | 0.012±0.017 | 7.3±0.23 |
| **SEM** | 0.32 | 0.235 | 0.76 |
| **p-value** | 0.0 | 0.0 | 0.0 |

Highly significant decrease were noticed between the levels of CP, Specific Activity of CP (SA), TP&Thiol and highly significant increase in the Carbonyl and Thiol/TP while Cyctatin-C levels were showed significant increase in the two studied groups ($p < 0.05$), table 3.

**Table 3:** Comparison of serum CP, Protein, SA of CP, Cyctatin-C,Carbonyl,Thioland Thiol/TP between CKD cases and control subjects

| Groups | CP U/L | Specific activity of CP | Protein g/dl | Carbonyl nmol/mg protein | Thiolµmol/L | Thiolµmol/g protein | Cyctatin-Cmg/dl |
|---|---|---|---|---|---|---|---|
| Controls (mean±SD) | 95.3±0.31 | 12.56±0.09 | 7.586±0.41 | 0.26±0.1 | 235.24±54.54 | 3.1±2.2 | 1.33±0.05 |
| Cases (mean±SD) | 70.2±0.11 | 12.4±0.23 | 5.66±0.7 | 0.787±0.113 | 189.08 ±36.42 | 3.3±9.01 | 2.1±0.003 |
| SEM | 0.65 | 0.55 | 0.146 | 0.025 | 12.89 | 0.1 | 0.11 |
| p-value | 0.0 | 0.025 | 0.0 | 0.0 | 0.001 | 0.0 | 0.043 |

Table 4 shown that the serum levels of Mo, Cd and Mg were significantly decreased while Mn showed significantly increase ($p < 0.05$)in CKD cases as compared to controls.

**Table 4:** Comparison of serum Mo, Cd, Mn& Mg between CKD cases and control subjects

| Groups | Molybdenum mg/L | Cadmium mg/L | Mnmg/L (mean ± SD ) | Mg mg/L ( mean ± SD ) |
|---|---|---|---|---|
| Controls (mean±SD) | 0.089±0.078 | 0.0187±0.002 | 20.285±1.424 | 23. 57±0.0238 |
| Cases (mean±SD) | 0.019±0.016 | 0.0129±0.008 | 24.389±2.93 | 18.59±0.0213 |
| SEM | 0.014 | 0.0014 | 0.009 | 0.003 |
| p-value | 0.00 | 0.0 | 0.0 | 0.0 |

The correlations results between the above factors in CKD patients were summarized in Table 5.Inverse significant correlation were noticed between the levels of T3andCyctatin-C,carbonyl,while, positive significant correlation between T3 and thiol, protein, Mn and Mgin the CKD group. T4 inversely significantly correlated with Cyctatin-C, carbonyl, Mn and Mg, and there were positive significant correlation between T4 and CP, thiol, protein in the CKD group. The positive significant correlation were noticed between the levels of TSH and CP,carbonyl, thiol and protein. Finally CP significantly positively correlated with Mn andMg and negatively significant correlation Cyctatin-C.

**Table 5:** Correlation between the measured parameters in CKD and control

| Correlation between | Patients | |
|---|---|---|
| | R | P |
| **T3 & CP** | 0.87 | 0.055 |
| **T3 & Carbonyl** | -0.83 | 0.023 |
| **T3 &Thiol** | 0.98 | 0.028 |
| **T3 & Protein** | 0.66 | 0.0 |
| **T3 &Cystatin C** | -089 | 0.032 |
| **T3 & Mo** | 0.3 | 0.056 |
| **T3 & Cd** | 0.51 | 0.059 |
| **T3 &Mn** | 0.99 | 0.02 |
| **T3 & Mg** | 0.59 | 0.012 |
| **T4 & CP** | 0.76 | 0.032 |
| **T4& Carbonyl** | -0.41 | 0.011 |
| **T4&Thiol** | 0.85 | 0.01 |
| **T4& Protein** | 0.88 | 0.03 |
| **T4&Cystatin C** | -0.78 | 0.045 |
| **T4& Mo** | -0.23 | 0.067 |
| **T4& Cd** | -0.67 | 0.089 |
| **T4&Mn** | -0.87 | 0.032 |
| **T4& Mg** | -0.95 | 0.021 |
| **TSH & CP** | 0.99 | 0.001 |
| **TSH & Carbonyl** | 0.69 | 0.044 |
| **TSH &Thiol** | 0.74 | 0.017 |
| **TSH & Protein** | 0.95 | 0.027 |
| **TSH&Cystatin C** | 0.56 | 0.098 |
| **TSH &Mo** | 0.45 | 0.089 |
| **TSH & Cd** | 0.76 | 0.067 |
| **TSH&Mn** | 0.76 | 0.065 |

| | | |
|---|---|---|
| **TSH& Mg** | 0.98 | 0.071 |
| **CP & Carbonyl** | -0.142 | 0.439 |
| **CP &Thiol** | -0.014 | 0.938 |
| **CP &Protein** | -0.236 | 0.194 |
| **CP & Mo** | 0.158 | 0.388 |
| **CP & Cd** | -0.089 | 0.629 |
| **CP&Mn** | 0.99 | 0.04 |
| **CP& Mg** | 0.95 | 0.032 |
| **CP&Cystatin C** | -0.87 | 0.025 |

## Discussion

Several studies have shown that thyroid hormones (T3 and T4) were mildly reduced while TSH level are usually normal or elevatedin patients with CKD[25,26], these results were agreed with the results obtained in this study (Table-2),Lim VS et al[27] reported that prevalence's of low T3 were 80% in non-hemodialysis patients and 43% inhemodialysis patients.Thyroid hormones are the most important factors involved in the regulation of the basal metabolic condition, as well as in the oxidative metabolism[28].Under normal conditions; the protective effect of thyroid hormone against oxidative stress can be explained by the function of antioxidants as a defense system[29].Oxidative stress plays a role in the pathogenesis of many chronic diseases including CKD[30-33].Ceruloplasmin acts as a very effective antioxidant[34],In the presence of reduced glutathione, CP has been demonstrated to protect DNA against oxidative stress [35]. Moreover, CP has been noted to protect proteins against ROS-induced carbonyl formation [36].According to Gutteridge and Quinlan [37], plasma CP together with iron-binding transferrin are the major plasma antioxidants although they represent no more than 4% of all plasma proteins. Chronic renal failure is an inflammatory disease and many researchers found that the levels of CP, an acute phase protein, should increase in these patients.Al-Salih et al[37] found that there were significant increase in CP activity in CKD patients. The possible factors contributing to the observed data for CP in this studym pry be that proteinuria and damage to this excreted protein due to the prevalent oxidative stress, alsoROS may disrupt copper binding to CP, thereby impairing its normal protective function while liberating the copper[38]. Thus, formation of uremic toxins and damage due to ROS may decrease CP levels in CKD patients[39].Levels of serum cystatin C was independently measured and compared with other factors. This study indicated that serum cystatin C levels were increased significantly in patients group compared to healthy control , these findings agreed with M. Sathishbabu[40],Khalid Bassiouny[41] and Fayrouz O. Selim[42].Serum cystatin C has been suggested to be an ideal endogenous marker of GFR. Theproduction of Cystatin C in the body is a stable process that is not influenced by renal conditions, increased protein catabolism, or dietetic factors. Moreover, it does not change with age or muscle mass like creatinine does. The biochemical characteristics of Cystatin C allow free filtration in the renal glomerulus, and subsequent metabolism and reabsorption by the proximal tubule[43,44,45],for these reasons, a number of studies stated that overt as well as subclinical thyroid dysfunctions could significantly alter serum cystatin C level [46-48].

This study demonstrated that the level of both serum total protein and protein thiols were decreased in patients group compared to control subjects. These results were agreed with KemidilIaiah et al[49].Kolagal Karanamet al [50],they found that protein thiols were decreased in patients with uremia ,they are sacrificed to quench ROS which are produced excessively in CKD patients, thus the levels of these proteins decrease in these patients.Totalthiol status in the body, especially thiol (SH) groups

present on protein are considered as major plasma antioxidant *in vivo* are present in a concentration higher than albumin and are major reducing groups present in the body fluids[51].Protein CO groups as biomarkers of oxidative stress has some advantages in comparison with the measurement of other oxidation products because of the relative early formation and the relative stability of carbonylated proteins. This study confirmed increased carbonyl formation in CRF patients.Jasmina et al[52] found that level of protein sulphydryl groups (P-SH) in plasma, which are important chain breaking "sacrificial" antioxidant, were markedly reduced in individuals with CRF compared to healthy subjects and carbonyl level as a marker of oxidative modification of proteins, increased in plasma of all chronic renal failure patients compared to healthy subjects.

Toxic metals have been studied extensively, however, there are few studies that have measured metals in blood and serum in a large population based cohort, where additional information is available for examining possible connections between the blood/serum distribution and physiological factors.Molybdenum (Mo) is an essential element for human and animals; however, high dietary intake of Mo can lead to adverse reactions. Cadmium (Cd) is harmful to health[53] and toxic metal causes damage to organs like kidney, lungs, bones, liver, brain and reproductive organ testes. But the most affected organ is kidney on cadmium exposure[54,55].This study aimed to examine the levels of Cd,Mo,Mn and Mg in the seraof CKDpatientsand compared to normal subjects ,results in this study indicated that the levels of Cd ,Mo and Mgwere decreased while Mn concentration were significantly increase in the sera of CKD patients compared to normal subjects.Subha, et al [55],revealed that the blood Cd concentration was higher in the end stage renal disease patients than healthy adults.Shinichi and Osamu [56], found that the levels of Mo were decreased in the sera of patients with CKD.

The correlation between serum thyroid hormones,cystatin C, CP and another parameters were done ,the results indicated that there were significant correlation between T3 and (cystatinC,carbonyl, thiol, protein),T4 and (CP,cystatinC,carbonyl,thiol,protein),TSH and (CP,carbonyl,thiol, protein).It is very much evident from the data of this study that thyroid hormone values disturbances are due to oxidative stress which impaired renal function.To summarize, Chronic renal failure affects, thyroid function in multiple ways, including low circulating thyroid hormone concentration, altered peripheral hormone metabolism, disturbed binding to carrier proteins. The data of this study supports that renal disease leads to significant changes in thyroid hormone levels that unlocks the significance of thyroid hormone quantification in chronic kidney disease patients.

## Conclusion

In conclusion, the results of the present study provide a clearer understanding of therelationship between some biochemical parameters includes thyroid hormones ,cystatin C , different antioxidantsand four trace elements in CKD subjects compared to healthy controls.The results suggests that CKD patients have an increased risk of subclinical hypothyroidismand prove the first demonstration that increased oxidative damage of serum proteins (measured as carbonyl ,thiol and CP content) correlates with the degree of renal insufficiency. Besides, the results presented also show that one of the features of CKD is the presence of signs of oxidative stress before hemodialysis.

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